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Potential of Ozonated Water at Different Temperatures to Improve Safety and Shelf-Life of Fresh Cut Lettuce
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ABSTRACT
The present study was carried out to determine the effect of ozonated water (2 mg L\(^{-1}\)) at different temperatures (4 °C and 15 °C) on the microbiological, color and sensory properties of lettuce. Cold ozone treatment (4 °C) significantly reduced the natural background microflora of lettuce. *Salmonella* Typhimurium and *Escherichia coli* inoculated on lettuce samples were insignificantly influenced by the temperature of water. During storage period at +4 °C for 14 days, the highest quality was observed from the samples treated with cold ozonated water. Ozone treatments did not affect the color properties and sensory quality of lettuce samples.

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KEYWORDS
Ozone; Temperature Effects; Submerging; Fresh Produce; Safety; Quality; Shelf-Life

Introduction
The consumption of raw or minimally processed forms of fresh fruits and vegetables has been increased in the last several years because of consumers’ trend to eat healthy food (Bermudez-Aguirre and Barbosa-Canovas 2013). Fresh and freshly cut produce can be vehicles of several pathogens such as *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* and *Shigella* spp., which have the ability to attach the surfaces of leafy vegetables and are responsible for foodborne outbreaks (Ramos et al. 2013). Hence, in the previous studies, carried out to decontaminate leafy vegetables, *Salmonella* Typhimurium and *E. coli* commonly used as test cultures at high inoculum levels (10\(^7\)–10\(^9\) CFU mL\(^{-1}\)) (Bermudez-Aguirre and Barbosa-Canovas 2013; Ding, Rahman, and Oh 2011). These microorganisms should be removed or inactivated in order to ensure the safety and quality of the product (Birmpa, Sfika, and Vantarakis 2013). New sanitizers or technologies have been investigated for the decontamination of the foods such as organic acids, ozone, ultraviolet light, ultrasound, cold plasma, and irradiation (Bermudez-Aguirre and Barbosa-Canovas 2013).

Effective sanitizers should be practicable, economically viable, acceptable to the consumer and legal (FAO/WHO 2008). There are many potential washwater sanitation approaches and each have some advantages and disadvantages. Chlorination is commonly used to disinfect fresh fruits and vegetables (Beuchat 1998). Antimicrobial activity of chlorine depends on variety of factors, such as the amount of free available chlorine in the wash-water, temperature, pH, treatment time, presence of organic matter, and vegetable tissue components (Beuchat 1998). There is also concern about residual by-products of chlorine, which could have carcinogenic properties (Allende et al. 2008).

In recent years, there is an increasing interest in ozone as an alternative sanitizer due to its high biocidal efficacy, wide antimicrobial spectrum, absence of by-products that are detrimental to health and the ability to generate it on demand, *‘in situ’*, without needing to store it for later use (Guzel-Seydim, Greene and Seydim, 2004; Pascual, Llorca, and Canut 2007). The use of ozone has been approved in the United States as effective disinfectant suitable for many applications such as food surface hygiene and preservation, equipment and food plant sanitation and reuse of waste waters (Alexopoulos et al. 2013; Kim, Yousef, and Khadre 2003; Ohashi-Kaneko et al. 2009; Park et al. 2008; Restaino et al. 1995; Sengun 2014; Smilanick, Margosan, and Mikota Gabler 2002; USDA 1997). Microbial inactivation effect of ozone depends on some factor such as the number and kind of contaminating microorganisms (Kim, Yousef, and Chism 1999), stability, solubility and concentration of ozone (Beiztoglou and Alexopoulos 2008; Rice...
1999; Sengun 2013, 2014), the presence of organic compounds and extreme pH variations of water (Ölmez and Akbas 2009), physiology of vegetable and contact time with ozone (Alexopoulos et al. 2013; Bermudez-Aguirre and Barbosa-Canovas 2013) and the temperature of ozonated water (Glowacz, Colgan, and Rees 2015a; Glowacz and Rees 2016). However, there are limited studies on the correlation between temperature of ozonated water and the removal of microorganisms or pesticides on vegetables and fruits (Glowacz and Rees 2016; Hwang, Cash, and Zabik 2001; Ikeura, Kobayashi, and Tamaki 2013).

Therefore, the objectives of the present study was: 1) to evaluate the use of ozonated water for the inactivation of Salmonella Typhimurium and Escherichia coli inoculated on fresh cut lettuce (Lactuca sativa), 2) to evaluate the effects of ozone submerging prior to storage period of lettuce in terms of microbiological, physical and sensory quality, 3) to compare the effects of aqueous solution of ozone produced water at different temperatures (4 °C and 15 °C).

Materials and methods

Bacterial culture

Salmonella Typhimurium ATCC 13311 and Escherichia coli ATCC 1103 were used in the study. The organisms were grown in Tryptone Soya Broth (TSB, pH 7.3 ± 0.2, Oxoid, Basingstoke, Hampshire, England). Stock cultures on Tryptone Soya Agar (TSA, pH 7.3 ± 0.2, Oxoid, Basingstoke, Hampshire, England) were stored at 4 °C and cultured for 24 h at 37 °C in TSB.

Sample preparation

Lettuce samples were purchased from a local supermarket in Izmir, Turkey and analyzed at the same day. After pre-cleaning, edible parts of lettuce leaves were washed with tap water and spin dried in a kitchen type spin drier for 2 min. Then lettuce leaves were cut with a sterile knife into small pieces of approximately 5 × 5 cm width. Ten-gram portions were weighed separately as sample units. To destroy the background microflora, 10 g of lettuce was placed into sterile glass plates and treated by UV light (Philips, TUV 15W) in a UV cabinet (Entkeimungsschrank, 220 V, Ernst Schuttjun Laborgerotebau, 3400, Gottingen) for 30 min (15 min for each side). Then lettuce samples were checked for the presence of Salmonella (BAM 2014) and E. coli cells (BAM 2002) before inoculation process.

Preparation of ozonated water

Pure water has the lowest ozone demand and ozone is more stable in low temperatures (Pascual, Llorca, and Canut 2007). Thus, pure water at 4 °C and 15 °C (± 2 °C) were used during ozone production. A 20 L plastic container was filled with water and two ozopen probes (Ozomax Inc., Quebec Canada) were dipped into water to produce ozonated water. The system, which has the maximum ozone production capacity of 2.0 ppm, was run till the required concentration of ozone was produced. Ozone concentration was measured by ozone test kit (0–2.3 mg L\(^{-1}\), Model OZ-2, Cat.No. 20644–00, Hach Lange, Düsseldorf, Germany).

Inoculation procedure

Lettuce leaves, which were found as negative for the presence of S. Typhimurium and E. coli, were used as analyze samples. Ten-gram of samples were inoculated by pipetting 0.1 mL of approximately \(10^{10}\) CFU mL\(^{-1}\) with S. Typhimurium and E. coli suspensions on the surface of the samples. Then inoculated samples were transferred into sterile jars to allow attachment of S. Typhimurium and E. coli cells, jars were stored at 20 °C for 30 min before exposing them to treatment.

Treatment of lettuce samples

Ten-gram of lettuce sample placed into sterile jar was combined with approximately 200 mL of pure water or ozonated water (at 4 °C or 15 °C), sufficient to cover the entire sample in the jar and agitated for 5, 10, and 15 min at room temperature. After decanting the treatment solutions, enumeration of S. Typhimurium and E. coli were done. Three replicates were carried out for each treatment.

Shelf-life of lettuce samples

To evaluate the effects of ozonated water on shelf-life of lettuce, uninoculated samples were prepared as given above. Then samples treated with ozonated water or pure water were placed in sterile stomacher bags (Interscience-190x300 mm) and stored at 4°C for 14 days. Untreated samples which were not treated with any kind of water were also stored under the same condition and used as control samples. Microbiological analysis (total mesophilic aerobic bacteria (TMAB) count, mold-yeast count), color
evaluation and sensory analysis were applied periodically during storage period.

**Microbiological analysis**

Ten-gram of lettuce was transferred in 90 mL of 0.1 % Peptone Water (PW) and homogenized for 2 min with a Stomacher (Lab-blender 400, Seward, London, UK). Appropriate 10-fold dilutions of samples were prepared in Peptone Water and plated in duplicate on/in growth media to estimate microbial counts.

For the initial loads of mesophiles on lettuce samples, pour plate method was used into Plate Count Agar (PCA, 7.0 ± 0.2, Oxoid CM325) and plates were incubated at a temperature of 35 °C for 24–48 h (BAM 2001a). Three replicates were carried out for each treatment and storage time.

The counts of mold-yeast were determined on Dichloran Rose Bengal Chloramphenicol Agar (DRBC, pH 5.6 ± 0.2, Oxoid CM0727) supplemented with Chloramphenicol (SR0078, Oxoid) at 25 °C for 3–5 days (BAM 2001b). Three replicates were carried out for each treatment and storage time.

To enumerate *S. Typhimurium* for treated samples, serially diluted homogenates were spread plated (0.1 mL in duplicate) on Bismuth Sulphite Agar (BSA, pH 7.6 ± 0.2, Oxoid CM0201) and plates were incubated at 37 °C for 48 h. Three replicates were carried out for each treatment.

Eosine Methylene Blue Agar (Levine) (EMB, pH 6.8 ± 0.2, Oxoid CM0069) was used for *E. coli* enumeration for treated samples and plates were incubated at 37 °C for 24 h. Three replicates were carried out for each treatment.

**Color properties**

Color parameters of treated and untreated samples were analyzed by using Konica Minolta CR-400 (Japan) colorimeter as described in Kramer and Twigg (1984). Lightness to darkness (L*), redness to greenness (a*), yellowness to blueness (b*), net color difference (ΔE*), chroma (C) and hue (H) values were quantified. Colorimeter was calibrated by using standard white plate (Y: 89,0; x: 0,3175 and y: 0,3346) before each measurement. Measurements were taken three times from three different parts (edge, middle and stem) of two lettuce leaves (total 18 measurements for each sample) and the average color values were calculated.

**Sensory analysis**

Sensory analyses were conducted by using scoring test with the attendance of 12 assessors (Altuğ-Onoğur and Elmacı 2011). Scoring characteristics were determined, scoring panel form (Figure 1) was established, and evaluation criteria were explained to the assessors during the training sessions. Sensory evaluations were conducted from 10:30 to 11:00 in the morning and from 14:30 to 15:00 in the afternoon. Five different samples were evaluated during each session, while each sample was evaluated 3 times in different sessions.

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Appearance</th>
<th>Texture</th>
<th>Flavor</th>
<th>Overall Impression</th>
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**Figure 1.** Scoring test form for the sensory evaluation of the lettuces.
**Statistical analysis**

Data of microbiological, color and sensory analysis were subjected to analysis of variance and Duncan’s multiple tests (SPSS 2004) to determine if there were significant differences (P < 0.05) between mean values.

**Results and discussion**

**Effect of ozonated water on S. Typhimurium and E. coli inoculated on lettuce**

In the study, efficiency of ozonated water on inactivation of *S. Typhimurium* and *E. coli* was investigated. Ozone is highly volatile and difficult to maintain in solution, thus, produced ozonated water was used immediately in the analyses. Residual ozone concentration was checked during experiments to verify ozone concentrations used.

The initial *S. Typhimurium* count (8.90 log CFU g⁻¹) on lettuce samples was significantly reduced by submerging applications (Table 1). The reduction achieved by ozone submerging for 5, 10 and 15 min were found in the range 2.44–2.57 log CFU g⁻¹ and 2.00–2.13 log CFU g⁻¹ for ozonated water at 4 °C and 15 °C, respectively. When pure water used at 4 °C and 15 °C, the counts of *S. Typhimurium* were reduced at around 1.00 log CFU g⁻¹ (Table 1). The results showed that the reduction effect of ozonated water was found significantly different from pure water (P < 0.05), while this effect was not changed depending on the treatment time and temperature of treatment solution used.

The initial count of *E. coli* (8.38 log CFU g⁻¹) was reduced in the range between 3.17–3.47 log CFU g⁻¹ and 2.93–3.30 log CFU g⁻¹ for ozonated water at 4 °C and 15 °C, respectively (Table 2). On the other hand, pure water submerging of lettuce sample was reduced the cell numbers for maximum 1.1 log CFU g⁻¹. These results showed that ozonated water was more effective than pure water for both test culture (P < 0.05). Ozone treatment caused higher reduction in the number of *E. coli* than *S. Typhimurium* (Tables 1 and 2) and *E. coli* cells were more sensitive than *Salmonella* cells for treatments applied by pure water (P < 0.05).

When the product has cut surfaces or tissue damages, ozone react with organic matter than react as an antimicrobial agent and thus the lethality of ozone decrease (Glowacz, Colgan, and Rees 2015a). Various microorganisms have different sensitivities to ozone (Alexopoulos et al. 2013). It was reported that the Gram-positive bacteria are more sensitive to ozone than the Gram-negative, while bacteria are more sensitive than mold and yeast (Cullen et al. 2010).

**Effect of ozonated water on background microflora of lettuce**

TMAB and mold-yeast counts of untreated lettuce samples were 3.9 ± 0.00 log CFU g⁻¹ and 4.13 ± 0.05 log CFU g⁻¹, respectively. Treatment of samples with ozonated water at 4 °C and 15 °C reduced the initial TMAB counts in the range of 2.31–2.75 log CFU g⁻¹ and 1.75–1.90 log CFU g⁻¹, respectively. Pure water (15 °C) treatment reduced TMAB counts of lettuce samples with a maximum of 0.9 log CFU g⁻¹ while cold pure water treatments caused significantly higher reduction rates (1.48–1.9 log CFU g⁻¹) than water at 15 °C (P < 0.05) (Figure 2). Mold and yeast counts of samples were decreased in the range of 1.52–2.15, 2.04–2.26, 1.63–2.54 and 0.41–0.90 log CFU g⁻¹ for treatments with ozonated water at 4 °C, ozonated water at 15 °C, pure water at 4 °C and pure water 15 °C, respectively (Figure 3).

In the previous study, TMAB counts of vegetables were reduced in the range between 0.09–1.25 log CFU g⁻¹ by ozonated water (at 0.5, 1.0 and 1.5 ppm for 3, 5 and 10 min) and high reduction level was detected in lettuce than in parsley (Sengun 2013). Koseki et al. (2001) reported that TMAB counts of lettuce samples reduced 1.5 log CFU g⁻¹ by 5 mg L⁻¹ aqueous ozone for 10 min application time. Similarly, Akbas and Olmez (2007), who reported a decrease of 1.7 log units in mesophilic bacteria for lettuce treated with ozone at 4 mg L⁻¹ for 2 min. Although the reduction rates of ozonated water used for rinsing fresh produce commonly reported as in the range between 1.0–5.9 log units, there are also studies which have been reported no decontaminating effects or a low reduction rates.

<table>
<thead>
<tr>
<th>Table 1. Effect of pure and ozonated water on <em>S. Typhimurium</em> reduction (Log10 CFU/g) on lettuce.</th>
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<td><strong>Time (min)</strong></td>
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*Data represent average value of three counts with their standard deviation. Means of the same row with different superscript letters differ significantly (P < 0.05) according to Duncan’s multiple range test.

<table>
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<th>Table 2. Effect of pure and ozonated water on <em>E. coli</em> reduction (Log10 CFU/g) on lettuce.</th>
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<td><strong>Time (min)</strong></td>
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*Data represent average value of three counts with their standard deviation. Means of the same row with different superscript letters differ significantly (P < 0.05) according to Duncan’s multiple range test.
Previously treated lettuce samples for 5, 10 and 15 min with ozonated water (at 2 mg L\(^{-1}\)) and pure water were stored at 4 °C for 14 days to determine the effects of treatments on TMAB and mold-yeast counts of lettuce during storage period. Untreated samples were also stored under the same conditions as control samples (Figures 2 and 3). During storage period, ozone and pure water treatments were found significantly different from each other (P < 0.05). Although the number of TMAB and mold-yeast of ozone treated samples were increased during storage period (1.31–1.80 log and 1.82–2.14 log increase in TMAB and mold-yeast count, respectively), it was still lower than the control samples (Figures 2 and 3). This finding confirms the results obtained by Beltran et al. (2005), who reported increased number of microbial counts for fresh-cut lettuce samples stored at 4 °C for 13 days, which was 1.8 log unit lower than control group. The results of our study showed that there was no significant difference between pure water treated and untreated (control) samples (P > 0.05) and ozone treatment were more efficient than pure water treatment (P < 0.05), while the effect of ozone treatment were not different for bacteria and fungi (P > 0.05).

In the previous study, lettuce and parsley samples treated with ozonated water (1.5 mg L\(^{-1}\) for 5 min) were stored at +4 °C for 15 days and at the end of storage period, the initial TMAB counts of lettuce samples were increased by 1.45, 1.79 and 1.15 log cycle for untreated, tap water treated and ozone

![Figure 2. TMAB counts of untreated and treated lettuce with pure and ozonated water during storage period. Error bars represent standard deviations.](image-url)
treated samples, respectively (Sengun 2013). Similarly, Wang, Feng, and Luo (2004) reported 1.0–1.5 log cycle increase in total aerobic plate count of ozone treated fresh-cut cilantro for 11 days of storage at 0 °C. Chauhan et al. (2011) reported 0.62 and 2.17 log cycle reductions for mold-yeast counts and standard plate count, respectively, for fresh-cut carrots treated with ozonated water (1:2 w v⁻¹; 200 mg O₃ h⁻¹) for 10 min. Ölmez and Akbas (2009) optimized the processing conditions for ozone treatment of lettuce samples as 2 mg L⁻¹ for 2 min, in terms of reducing the microbial load and maintaining sensory quality during cold storage. All these studies showed that using ozonated water prior to storage is efficient method in reducing microbial counts on fresh produce (Alegria et al. 2009; Ding, Rahman, and Oh 2011; Ketteringham et al. 2006; Klaiber et al. 2004).

**Effects of ozonated water on color properties**

Visual quality loss during storage of lettuce samples was determined by monitoring color changes. Lettuce samples treated with ozonated water (at 2 mg L⁻¹) and pure water for 15 min were monitored for color analysis. It was found that ozonated water and pure water treatments did not cause any differences on the color properties of the samples and there was no difference (P > 0.05) between the color of the both treated and untreated samples (Figure 4). Similar result was also reported by various researchers for fresh produce (Glowacz, Colgan, and Rees 2015a, 2015b).
Both treated and untreated (as control) samples were stored at 4 °C for 14 days and changes in the color properties of the samples were also observed during the storage period (Figure 5). It was found that although the L* value (lightness) tended to rise with the storage period for all of the treatment conditions as well as control, this increase was not significant (P > 0.05). This result showed that L* value was not affected by the type and temperature of the treatment water and did not change with the storage at 4 °C for 14 days. a* (greenness), b* (yellowness) and C* (chroma) values of the samples were not affected neither treatment type and temperature nor storage at 4 °C for 14 days (P > 0.05). When the samples were evaluated in terms of ΔE* value, which indicates the net color difference, it was found that this value tended to decrease with the storage period for all of the treatment conditions as well as control, whereas it was not significant (P > 0.05). Although no important increase was observed in the hue of the samples (P > 0.05) during the storage period, it was found that h value of the samples at the end of the storage was significantly higher than the beginning (P < 0.05).

The results of this study showed that treatment with pure and ozonated water (2 mg L⁻¹) at different temperatures (4 °C and 15 °C) has not any effect on the color properties of the lettuce samples. Bermudez-Aguirre and Barbosa-Canovas (2013) reported that ozone treatment (5 mg L⁻¹) caused loss in the characteristic green color and leafy structure of lettuce showed a white coloration (increase in L* value). On the other hand, when ozone concentration was 1 mg L⁻¹, no change was observed in the color properties of lettuce (Baur et al. 2004).

**Effects of ozonated water on sensory quality**

Lettuce samples treated with ozonated water (at 2 mg L⁻¹) and pure water for 15 min were selected for sensory scoring test. Samples were evaluated according to their appearance, texture, flavor, and overall impression. It was found that the samples showed alterations (P < 0.05) in terms of sensory quality depending on the treatment water used (Figure 6). Although Ölmez and Akbas (2009) reported that ozone treated samples gained better scores in all sensory attributes compared with water treatments, in our study ozonated water caused decrease in sensory scores of appearance, texture, and overall impression (P < 0.05) while flavor was not affected by treatment solution.

The sensory properties of the samples stored at 4 °C were also observed for 14 days (Figure 7). During the storage period, both treated and untreated (as control) lettuce samples started to become dull and lost their appearance quality (Figure 7). After 2 days of storage, discoloration and browning was observed on the samples, this quality loss continued by storage and after the 4th day appearance scores decreased below “3” (Figure 7). Storage also significantly affected the textural and flavor quality of the samples (P < 0.05). Texture loss during storage is a serious problem for fresh produce (Glowacz, Colgan, and Rees 2015b). Hence, it is not surprising to observe texture loss in control and lettuce samples exposed to ozone and pure water after 14 d of storage at 4 °C. From the beginning to the 2nd day of the storage, all of the samples started to lose their freshness and crispness as well as their typical flavor. Especially decrease in flavor scores continued by the storage period and at the 10th day, flavor scores decreased below “3” (Figure 7). Besides, overall impression of the samples was also decreased by the storage (P < 0.05). At the beginning of storage, overall impression score of the samples was “4-good”, whereas this score decreased continuously and after the 14th day, it become around “2-poor” (Figure 7). At the end of the storage period, it was observed that
the samples treated with ozonated water at 4 °C had the minimum quality loss among all of the treatment conditions. Although the samples treated with ozonated water at 4 °C had the lowest appearance, texture, flavor and overall impression scores at the beginning of the storage, they were found as the least influenced samples by the storage period.

It was reported that there were no differences in color, texture and freshness in fresh-cut iceberg lettuce, parsley, cucumber, zucchini and rocket leaves.

Figure 5. Changes in color properties (L*, a*, b*, ΔE*, C*, h values) of untreated and treated lettuce with pure and ozonated water during storage period. Error bars represent standard deviations.
washed with ozonated water (Beltran et al. 2005; Glowacz, Colgan, and Rees 2015b; Martínez-Sánchez et al. 2006; Sengun 2013). However the results of our study showed that treatment procedure (using pure water or ozonated water) caused some changes in the sensory properties of lettuce samples. On the other hand, treatment type did not affect the changes of sensory quality during storage period (P > 0.05) and it was determined that decrease in the sensory quality of the samples showed similar trends except ozone treatment at 4 °C, which cause less quality loss. Baur et al. (2004) and Baur et al. (2005) also reported that the storage caused decrease in texture/crispness and flavor of the iceberg lettuce without affecting the treatment.

**Conclusion**

Ozonation of water used for submerging shredded lettuce helps to reduce the microbial load while inducing the safety level of the product by showing antimicrobial effect on pathogenic microorganisms. The highest quality was observed from the samples

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**Figure 5.** (Continued).

**Figure 6.** Sensory scores of lettuce samples treated with pure and ozonated water (2 mg L$^{-1}$).

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Beltran et al., Glowacz, Colgan, and Rees, Martínez-Sánchez, Sengun, Baur, Baur et al.
treated with cold ozonated water during storage period. Cold water treatments caused higher reduction levels in the number of microorganisms than treatment with water at 15 °C. Moreover, application of ozonated water produced from cold water could be more useful in protecting the quality of produce during storage period. Hence, ozonated water could be a good alternative for submerging vegetables not only for providing microbial quality but also by keeping the sensory quality of the product with

Figure 7. Changes in sensory scores of untreated and treated lettuce with pure and ozonated water during storage period. Error bars represent standard deviations.
absence of by-products, which are detrimental to health.

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